
# Seven Types of Tunic Cells in the Colonial Ascidian Aplidium yamazii (Polyclinidae, Aplousobranchia) : Morphology, Classification, and Possible Functions

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# Seven Types of Tunic Cells in the Colonial Ascidian *Aplidium yamazii* (Polyclinidae, Aplousobranchia): Morphology, Classification, and Possible Functions

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**ABSTRACT**—Seven types of tunic cells and multicellular vesicles are described in the tunic of the polyclinid ascidian *Aplidium yamazii*: spherical tunic cells (t. c.), phagocytic t. c., elongated t. c., filopodial t. c., micro-granular t. c., morula-like t. c., and macro-granular t. c. The spherical tunic cells are characterized by their spherical cell bodies filled with round granules. Some of these cells are located on the outside of the tunic, and they are probably released from the tunic. The phagocytic tunic cells are irregularly shaped and are motile. They contain phagosomes and vesicles laden with round granules, which are probably derived from the contents of the phagosomes. It is thought that the phagocytic tunic cells filled with the granules become the spherical tunic cells that are released from the tunic. The elongated tunic cells have thin cell bodies with long cellular processes extending from them. These processes appear to connect with each other and possibly form a contractile network in the tunic. The tunic vesicle is a hollow multicellular vesicle composed of thin flattened cells and cuboidal granular cells. The cuboidal cells of the tunic vesicle often contain a large amount of rough endoplasmic reticulum, indicating a high level of protein synthesis, and they may secrete some components of the tunic. The functions of the other tunic cells, i.e., filopodial tunic cell, micro-granular tunic cell, morula-like tunic cell, and macro-granular tunic cell, are not clear.

## INTRODUCTION

Ascidian bodies are completely covered with the tunic, which is a leathery or gelatinous matrix containing cellulosic fibrils [1, 20]. There are several different kinds of tunic cells distributed within the tunic. In this sense, the ascidian tunic is not merely a covering, but is a living tissue performing some biological functions. In colonial species, the tunic is also a shared tissue among the clonal individuals and possibly involves some events that are characteristic of colonial organisms (e.g., budding of zooids [14, 15], coordination of zooids [10], allorecognition between colonies [18], *etc.*).

Tunic cell morphology and classification have been described for a few groups of colonial ascidians. In botryllid ascidians, which have an interzooidal (common) vascular system, there are only two or three types of tunic cells [8, 9, 22], and hemocytes infiltrating from the vascular system are involved in some events occurring in the tunic, such as the allogeneic rejection reaction between colonies [7, 19]. On the other hand, aplousobranchian species, lacking an interzooidal vascular system, usually possess various types of tunic cells. For instance, six types of tunic cells are recognized in the aplousobranchian didemnid *Leptoclinides echinatus* [5]. In the species without tunic vessels, it is difficult for the hemocytes to respond quickly to the events occurring in the tunic, because they have to migrate for a long distance. These aplousobranchian species are, therefore, likely to have more types of tunic cells for dealing with these events than do the species with an interzooidal vascular system, such as botryllids.

Recently, we reported that a particular type of tunic cell shows phagocytic activity in the aplousobranchian polyclinid *Aplidium yamazii* [6]. Another type of tunic cell probably has another function in the tunic. In order to understand the various biological events in the tunic, it is necessary to describe and classify the tunic cells in this species. The present study deals with the classification of the tunic cells of *A. yamazii* based on light and electron microscopical observations. We also discuss the possible functions of some of these tunic cells.

## MATERIALS AND METHODS

### *Animals*

The colonies of *Aplidium yamazii* were collected in Nabeta Bay, Shimoda (Shizuoka Pref., Japan). They were attached to glass slides with cotton thread and were reared in culture boxes immersed in Nabeta Bay. The colonies grew and spread on the glass slides.

### *Light and electron microscopy*

A living colony was sliced transversely as thin as possible (about

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0.5 mm or less) with a razor blade and mounted with seawater. The live specimens were observed under a light microscope equipped with Nomarski differential interference contrast (DIC) optics.

A colony was cut into pieces and fixed in 2.5% glutaraldehyde-0.1 M sodium cacodylate-0.45 M sucrose (pH 7.4) for 2 hr on ice. These colony pieces were then washed in 0.1 M sodium cacodylate-0.45 M sucrose (pH 7.4), and postfixed in 1% osmium tetroxide-0.1 M sodium cacodylate (pH 7.4) for 1 to 1.5 hr on ice. The specimens were dehydrated through an ethanol series, cleared with *n*-butyl glycidyl ether, and embedded in low viscosity epoxy resins. Thick sections were stained with toluidine blue for light microscopy (LM). For transmission electron microscopy (TEM), thin sections were stained with uranyl acetate and lead citrate, and they were examined in a Hitachi HS-9 transmission electron microscope at 75 kV.

*Recording of cell motility*

The motility of tunic cells was recorded using a time-lapse videocassette recorder AG-6010 (National, Japan). A colony was sliced with a razor blade, mounted with seawater, and observed by a microscope equipped with Nomarski DIC optics and a video camera WV-1800 (National, Japan). The recording was performed about 1/60 of the actual speed.

*Staining of microfilaments with phalloidin-fluorescein isothiocyanate (FITC)*

Microfilaments were visualized in colony slices by labeling with phalloidin-FITC. A colony was cut into slices with a razor blade. The slices of the colony were fixed with 3.5% formaldehyde in $Ca^{2+}$-free artificial seawater (CFSW; Jamarine Lab., Japan) for 10 min, permeabilized with 0.1% Triton X-100 in CFSW for 5 min, and washed with phosphate buffered saline (PBS). They were incubated with 1 $\mu$g/ml phalloidin-FITC (Sigma) in PBS for 30 min and then were rinsed extensively with PBS. The specimens were observed under a microscope equipped with epifluorescence and Nomarski DIC optics.

## RESULTS

*Morphology and classification of tunic cells*

We identified seven types of tunic cells based on their morphology: spherical tunic cells (t. c.), phagocytic t. c., elongated t. c., filopodial t. c., micro-granular t. c., morula-like t. c., and macro-granular t. c. The micro-granular and macro-granular t. c. are fewer in number than the other types. There are also hollow multicellular vesicles, called tunic vesicles, in the tunic.

**Spherical t. c.** (Fig. 1): These are one of the prominent tunic cell types. They are characterized by a spherical cell shape and round granules (about 1 to 1.5 $\mu$m in diameter) that occupy the bulk of the cells (Fig. 1, A and B). The granules show a variety of electron densities, and each of them is contained in a vesicle. The tunic cuticle often surrounds the cells distributed near the cuticle (Fig. 1A). This indicates that these cells are present outside the tunic. Some of the spherical t. c. appear to be released from tunic surface (Fig. 1, C and D).

**Phagocytic t. c.** (Fig. 2): This is the most prominent type of tunic cell, being distributed throughout the tunic. These cells are characterized by phagocytic activity, as described previously [6]. The phagocytic tunic cells often have phagosomes containing disorganized structures, and some of them also engulf other tunic cells. They also contain round granules, 1.5 $\mu$m or less in diameter. The granules of 1 to 1.5 $\mu$m in diameter are similar in morphology to those of the spherical t. c. described above. The cells containing no or few granules have an irregularly shaped cell body with numerous filopodia, whereas the cells containing many granules have a relatively thicker cell body with fewer filopodia. Some of the phagocytic tunic cells filled with granules are similar in morphology to the spherical t. c. (Fig. 2C).

**Elongated t. c.** (Fig. 3): These cells are flat and extend cellular processes that are often 70 $\mu$m or greater in length. They often have vacuoles, but no prominent granule in the cytoplasm. The cellular processes appear to contact those of neighboring elongated t. c. and probably form a network of these cells.

**Filopodial t. c.** (Fig. 4): These cells are usually distributed near the tunic cuticle. Under Nomarski DIC optics, the cells have a stellate shape and radiate filopodia (Fig. 4A). They often have vacuoles, but no prominent granules. Because of the limited information from thin sections, it is difficult to define the filopodial tunic cells in TEM. There are nongranular amoeboid tunic cells near the tunic cuticle, and they are the most probable candidates for the filopodial t. c. (Fig. 4B).

**Micro-granular t. c.** (Fig. 5): These cells are characterized by electron-dense granules of about 0.2 $\mu$m in diameter. They are irregularly shaped and protrude pseudopodia. It is impracticable to discriminate between this type of cell and the phagocytic t. c. in LM.

**Morula-like t. c.** (Fig. 6): These cells closely resemble morula cells, which are a kind of hemocyte that has been described in many other ascidians [21]. The greater part of these cells is occupied by several vacuoles filled with electron-dense materials (Fig. 6A). The cells that are distributed close to the epidermis of zooids are often elliptically shaped, whereas the others are round.

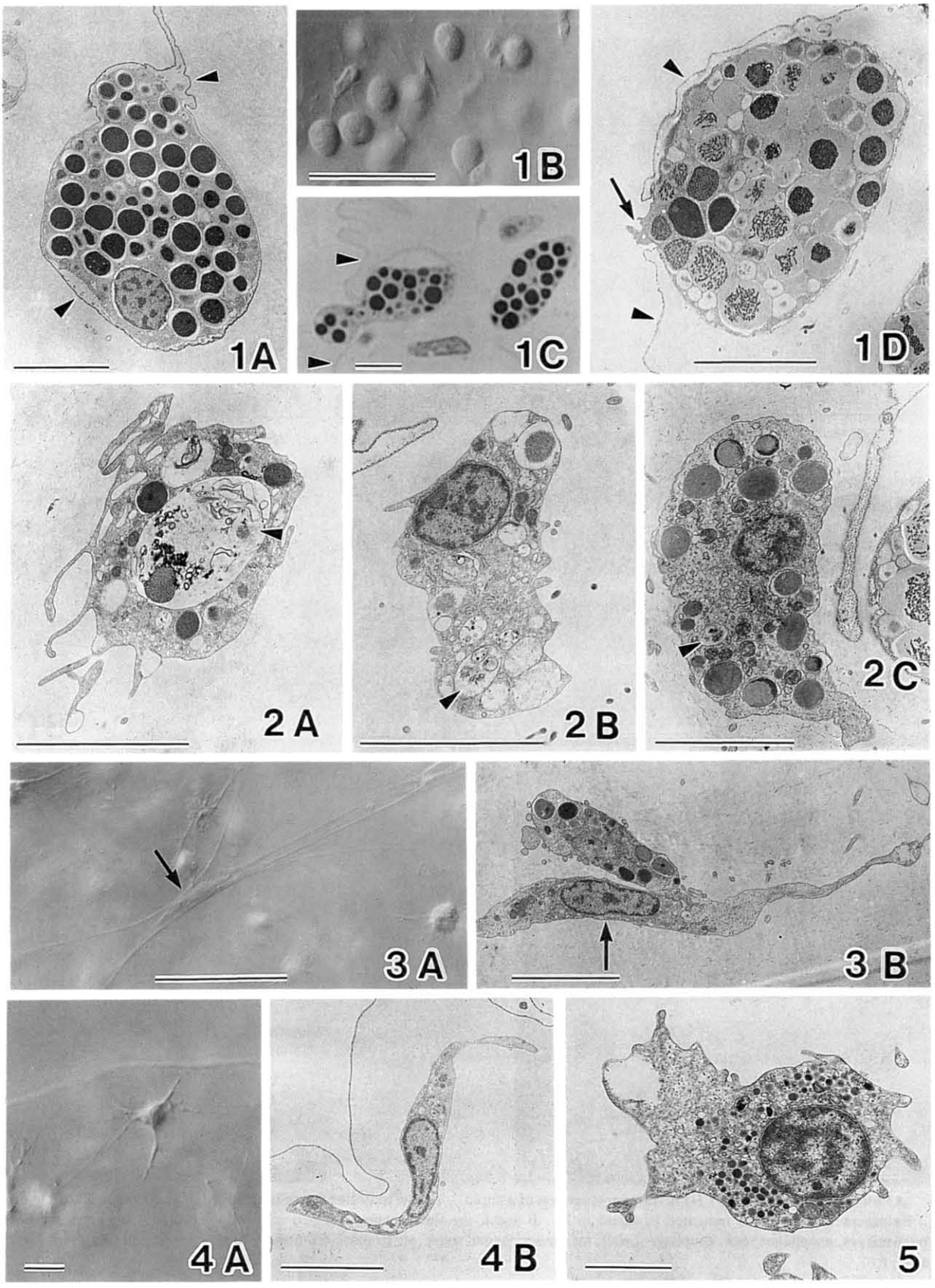
FIG. 1. Spherical tunic cells. Arrowheads indicate the tunic cuticle. A) The cell surrounded by the tunic cuticle. B) The cells in a live specimen under Nomarski DIC. C) The cell, half-released from the tunic, in the thick sections stained with toluidine blue. D) A part of the cell projecting out from the tunic (arrow). Scale bars=5 $\mu$m (A, C and D) and 50 $\mu$m (B).

FIG. 2. Phagocytic tunic cells. Arrowheads indicate phagosomes containing disorganized structures. Scale bars=5 $\mu$m.

FIG. 3. Elongated tunic cells (arrows) in a live specimen (Nomarski DIC, A) and in thin section (TEM, B). Scale bars=50 $\mu$m (A) and 5 $\mu$m (B).

FIG. 4. A filopodial tunic cell in a live specimen (Nomarski DIC, A) and a possible candidate in thin section (TEM, B). Scale bars=10 $\mu$m (A) and 5 $\mu$m (B).

FIG. 5. A micro-granular tunic cell. Scale bar=2 $\mu$m.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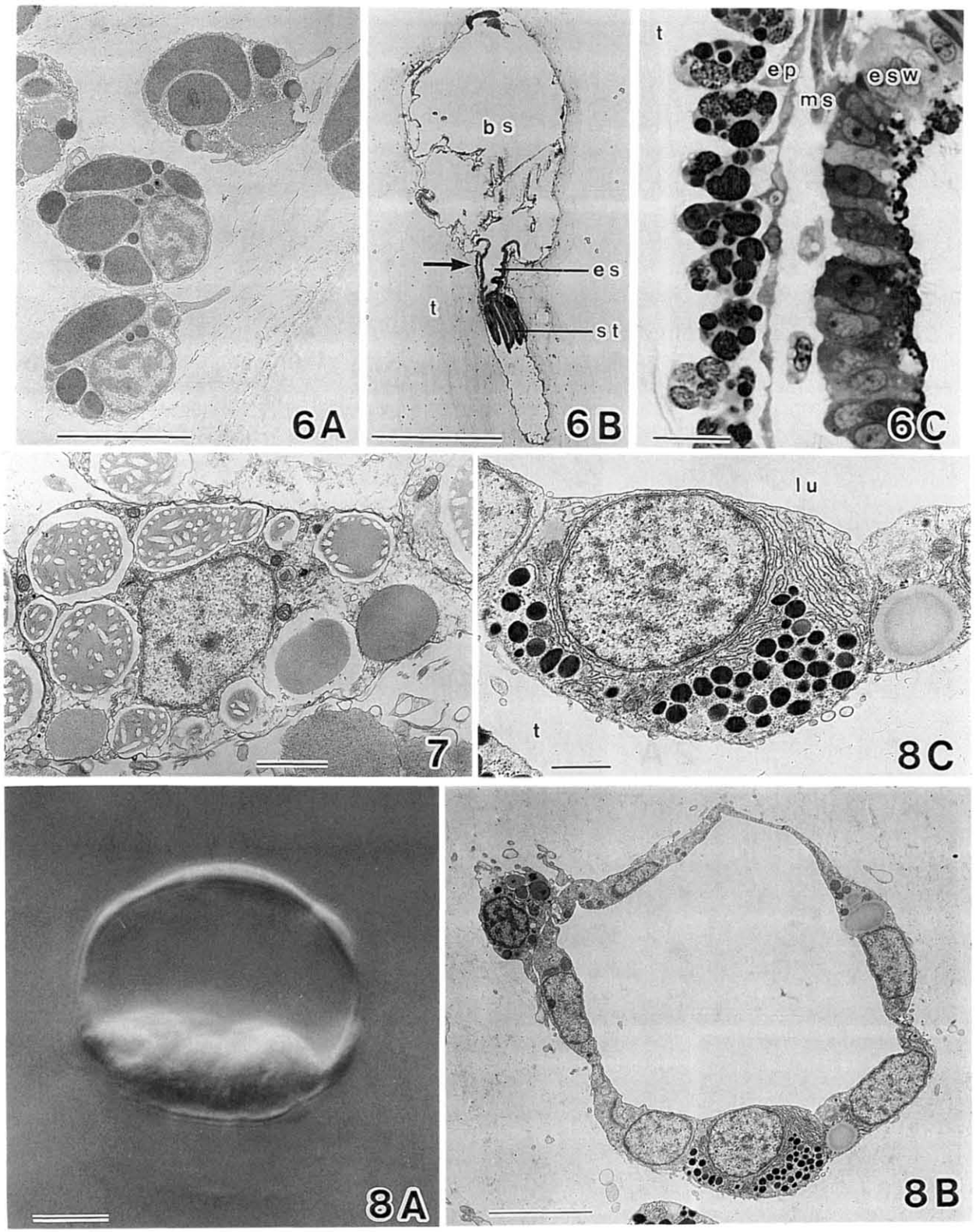


FIG. 6. A) Morula-like tunic cells. B) A transverse section of a zooid. Arrow indicates the cells arranged in a row adjacent to the epidermis. C) Enlargement of the area indicated by arrow in B. B and C are thick sections stained with toluidine blue. bs, branchial basket; ep, epidermis; es, esophagus; esw, esophageal wall; ms, mesenchymal space; st, stomach=t, tunic. Scale bars=5 $\mu$m (A), 50 $\mu$m (B), and 10 $\mu$m (C).

FIG. 7. A macro-granular tunic cell. Scale bar=2 $\mu$m.

FIG. 8. Tunic vesicles. A) Nomarski DIC in a live specimen. B) TEM. C) Cuboidal cells of tunic vesicles (enlargement of B). lu, lumen of tunic vesicles; t, tunic. Scale bars=10 $\mu$m (A), 5 $\mu$m (B),, and 2 $\mu$m (C).

Many morula-like t. c. surround the anterior-most abdominal epidermis that covers the esophagus (Fig. 6, B and C). These cells are arranged in a row adjacent to the epidermis. The area of this aggregative cell distribution is limited, and the cells do not encircle this area like a collar.

**Macro-granular t. c.** (Fig. 7); These are distributed close to the epidermis of the zooid and usually form a cell mass. They are characterized by round vacuoles each of which contains a large granule approximately 2 $\mu$m in diameter. The granule is not homogeneous, and there are many electron-lucent areas lined with electron-dense substances in each granule.

**Tunic vesicle** (Fig. 8): There are hollow multicellular vesicles in the tunic. Simple epithelial cells form the wall of the vesicle, and they consist of at least two cell types: thin flattened cells and cuboidal granular cells. The flattened cells contain some mitochondria and occasionally a few moderately dense granules (about 1 $\mu$m in diameter). The cuboidal granular cells are characterized by electron-dense granules of 0.3 to 0.4 $\mu$m in diameter and a large amount of rough endoplasmic reticulum (RER) (Fig. 8C). In both cell types, we did not find a prominent basal lamina on either the tunic side or the inner side.

*Distribution and composition of tunic cells*

Tunic cells of each type are not distributed homogeneously in the tunic. Preliminary analysis of the distribution and composition of each cell type was carried out in the thick sections stained with toluidine blue (Table 1). The seven types of tunic cells described here were grouped into three types, because some cell types were not classified perfectly in the thick sections. Namely, "amoeboid type" includes phagocytic, filopodial, micro-granular, and elongated t. c., and "morula-like type" includes morula-like and macro-granular t. c. The numbers of each cell type were determined in three different areas of the tunic: beneath the tunic cuticle; around the zooid (abdomen), including the area where morula-like t. c. are aggregatively distributed; and the peripheral part of the colony. The spherical t. c. was relatively abundant in the subcuticular area and rare around the zooid. In contrast, many of the morula-like t. c. were found around the zooids. The epidermal vesicles were often distributed around the colony periphery.

TABLE 1. Differential cell counts of tunic cells in three different tunic area

| Cell type[2] | Number (%) of cells in[1]: Subcuticle | Abdomen | Periphery |
|---|---|---|---|
| Spherical | 37 (14) | 3 (1) | 5 (7) |
| Amoeboid | 217 (83) | 190 (72) | 62 (86) |
| Morula-like | 9 (3) | 70 (27) | 1 (1) |
| Epidermal vesicle[3] | 0 (0) | 1 (<1) | 4 (6) |
| Total | 263 | 264 | 72 |

[1] Subcuticle=subcuticular area (within 50 $\mu$m below tunic cuticle) around branchial siphon; Abdomen=around the abdomen of zooids (within 50 $\mu$m along abdominal epidermis); Periphery=peripheral parts of the colony, excluding subcuticular area.

[2] The three cell types listed here represent all seven types described in the text because of the difficulty of classification in thick sections (see text).

[3] Each epidermal vesicle is counted as a single cell, although it is a multicellular structure.

*Cell motility and cellular microfilaments*

Many of phagocytic t. c. migrate actively in the tunic, and some others that extend filopodia radially do not migrate. Filopodial t. c. that are distributed near the cuticle usually repeatedly contract and expand, but do not change position in the tunic, whereas elongated t. c. rarely contract. Spherical t. c. and morula-like t. c. are not motile. The tunic vesicles do not migrate in the tunic, and each of them repeatedly contracts and expands. Under a light microscope, it is very difficult to distinguish the micro-granular t. c. and the macro-granular t. c. from some of the phagocytic t. c. and the morula-like t. c., respectively, and thus, the cell motility of these two cell types is uncertain in this study. The speed of cell migration could not be measured because of three-dimensional migration of the tunic cells in the tunic slices.

Phalloidin-FITC stains the cellular microfilaments (espe-

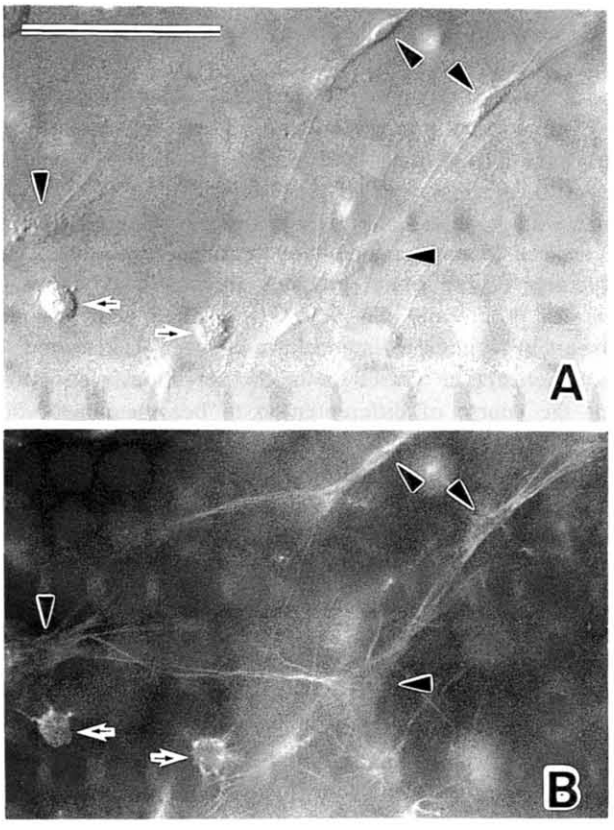


FIG. 9. Paired images of tunic slices stained with phalloidin-FITC (Nomarski DIC for A, fluorescent microscopy for B). Arrow, phagocytic tunic cell; arrowhead, elongated tunic cell. Scale bar=50 $\mu$m.

cially the filopodia) of the phagocytic t. c., filopodial t. c. and elongated t. c. (Fig. 9). The filopodia of the elongated t. c. appear to connect with one another, thereby possibly forming a cellular network in the tunic. The other tunic cells show only weak fluorescence at their cell periphery.

## DISCUSSION

The present study describes seven types of tunic cells and a type of multicellular vesicle in the colonial ascidian *Aplidium yamazii*. The possible functions of some of the tunic cells are discussed, based on morphological findings.

Spherical t. c. are characterized by their spherical cell shape and are filled with round granules. Many spherical t. c. are distributed in the subcuticular area of the tunic, and some of them are released from the tunic. It is not known how they move to the outside of the tunic, since the time-lapse video recording shows their immotility. Release of tunic cells has been reported in a solitary ascidian, *Ciona intestinalis*: the large granule cell in the adult tunic [2] and the large single vacuole cell and the type B cell in the metamorphosing embryo [11, 12]. According to these reports, these tunic cells are presumed to migrate from the mesenchymal space between the epidermis and the peribranchial epithelium, cross the epidermis, and finally reach the outside of the tunic cuticle. These excreted cells in *C. intestinalis* have a large vesicle containing electron-dense materials, and they differ in structure from the spherical t. c. that are multigranular. It is possible that the excreted cells of *C. intestinalis* and *A. yamazii* originate from different tissues, respectively.

Phagocytic t. c. are characterized by phagocytic activity and have phagosomes and round granules. Peroxidase activity was demonstrated exclusively within the vesicles laden with granules, and this suggests that the granules may be derived from the contents of the phagosomes [6]. The structures of the round granules are similar to those of spherical t. c., and phagocytic t. c. filled with granules appears to be intermediate in form between phagocytic t. c. and spherical t. c. Based on these observations, we suppose that the course of differentiation to become phagocytic/spherical t. c. is as follows: 1) The young phagocytic tunic cell has a thin cell body and numerous protruding filopodia. 2) The round granules are produced from the contents of phagolysosomes. 3) In accordance with the increase of granules, the cell body becomes thicker and roundish. 4) The cells that are full of granules differentiate into spherical t. c. and then are excreted from the tunic as waste.

The long cellular processes of elongated t. c. appear to connect with each other to form a cellular network in the tunic. Mackie and Singla [10] reported a network of cells in the tunic of two colonial species, *Diplosoma listerianum* and *D. macdonaldi*. The cells forming the network are called myocytes; they stain with NBD-phallacidin and show contractility. The myocytes are usually multipolar with long cellular processes, and the net of myocytes itself is supposed to conduct impulses that trigger its contraction, according to electrophysiological studies [10]. In the tunic of the *Diplosoma*, Mackie and Singla [10] also described filopodial cells that are restricted to the surface layer of the tunic. The filopodial cell in the *Diplosoma* probably corresponds to the filopodial t. c. in *A. yamazii*. In *Diplosoma*, the filopodial cells and the myocytes are distributed separately in the tunic. According to Mackie and Singla [10], "filopodial cells are restricted to the surface layer of the tunic, while the myocyte lie deeper." This is probably true for filopodial t. c. and elongated t. c. in *A. yamazii*. In *Leptoclinides echinatus*, six types of tunic cells are described by means of LM for paraffin sections and by scanning electron microscopy [5], and the "elongated tunic cell" in this species is similar in cell shape to the elongated t. c. in *A. yamazii*.

Tunic vesicles (or epidermal vesicles) have been reported in two colonial ascidians: *Aplidium* (=*Amaroucium*) *constellatum* [3, 4] and *Polycitor proliferus* [16, 17]. These studies reported that tunic vesicles are the result of evagination from the epidermis of zooids during the late embryonic stage. Oka and Usui [17] assumed, on the basis of their histological examinations, that the tunic vesicles are constantly formed, even in adult colonies. They also supposed that the tunic vesicles in *P. proliferus* secrete some adhesive substance to fix the colony to the substratum. In *A. yamazii*, the cells of tunic vesicles contain a large amount of RER and many granules, suggesting synthetic and secretory activity.

In general, tunic cells are thought to originate from hemocytes that pass through the epidermis. In *Aplidium* (= *Amaroucium*) *yamazii*, Nakauchi [13] has reported that "many tunic cells surrounding the neck of the abdomen become free from the zooid, and they migrate into the tunic" during the last stage of budding. In the present study, we have also found that many morula-like t. c. aggregate in the tunic around the epidermis covering the esophagus (neck of abdomen). This possibly indicates that morula-like t. c. originate from morula cells of hemocytes that have passed through the epidermis at this area. Since morula-like t. c. are, however, a kind of differentiated cell, they will not differentiate into other types of tunic cells, such as phagocytic t. c. or elongated t. c. This observation, therefore, cannot support the idea that these cell aggregation is a source of all types of tunic cells in *A. yamazii*. The origin of each type of tunic cell still remains unresolved.

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